CHARACTERISATION OF AN EPIDEMIC STRAIN OF *KLEBSIELLA* AND ITS VARIANTS BY COMPUTER ANALYSIS

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SUMMARY. An outbreak of hospital-acquired klebsiella infection was investigated by means of six different typing schemes. Interpretation of the information generated by these schemes proved difficult and allowed only tentative conclusions to be reached concerning the origin of some strains. Consequently, the results were subjected to computer analysis by means of a numerical taxonomic programme adapted for the purpose. A dendrogram was constructed giving the percentage similarity between strains. It established the source of four atypical strains and showed that three of them were derived from the original clone. This form of numerical epidemiological analysis could have useful applications in the investigation of hospital-acquired infection.

INTRODUCTION

The value of epidemiological typing methods in the investigation and control of many microbial diseases, community-acquired and hospital-acquired, has long been well established.

A marked change has taken place in the aetiology of hospital-acquired infection during the last three decades. Hospital sepsis, once synonymous with staphylococcal infection, is now more likely to be due to one of a variety of gram-negative bacteria. The typing methods available for the investigation and control of these have not yet been fully evaluated and several different methods are currently in use.

We have recently investigated an outbreak of hospital-acquired infection caused by a resistant strain of *Klebsiella* which was initially believed to be readily identifiable. However, during the course of the epidemiological investigation, patients were encountered who were infected with strains very similar to, but not identical with, the index strain. Whether the latter strains were totally distinct from the index strain, or were variants arising from it, was unclear. Six different typing systems, some now in routine use, have been

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applied to the strains associated with this incident and to unrelated strains in an attempt to establish the origin of the atypical strains. To assist the evaluation, a computer analysis of the data has been made. As far as we are aware, this is the first time that this kind of analysis has been used for epidemiological purposes.

MATERIALS AND METHODS

Biochemistry. Isolates were initially tested by the API 20E system (API Laboratory Products Ltd, Invincible Road, Farnborough, Hampshire GU14 7QM) and identification of *Klebsiella pneumoniae* or *oxytoca* made from the profile number; *K. oxytoca* differed by being indole positive. Other tests performed included motility, growth in potassium cyanide, methyl-red reaction (MR), malonate utilisation, gas production from glucose and dulcitol fermentation (Cowan and Steel, 1974). Isolates were incubated for 4 weeks to assess fermentation reactions and gas production.

Antibiotic sensitivity was initially tested by disc diffusion (Stokes and Waterworth, 1972) of sulphonamide, trimethoprim, ampicillin, nalidixic acid, nitrofurantoin and gentamicin. Later, tests were made for sensitivity to tetracycline, chloramphenicol, cephalothin, mecillinam, neomycin, paromomycin, kanamycin, sisomicin, tobramycin, amikacin, streptomycin and spectinomycin. The minimum inhibitory concentration (MIC) of gentamicin was measured for all strains with a multipoint inoculator, an inoculum of 10^3 bacteria and nutrient-agar plates incorporating dilutions of gentamicin. After overnight incubation the end point was determined as the lowest concentration at which there was no growth. The mechanism of aminoglycoside resistance was inferred from the resistance pattern (Shannon, Phillips and King, 1978).

Capsule typing was done at the Public Health Laboratory, Coventry, by an immunoelectrophoretic method (Palfreyman, 1978).

Bacteriophage typing was done in the Division of Hospital Infection, Central Public Health Laboratory, Colindale, by an experimental method in which 19 typing phages were used.

Klebicine typing. Sensitivity to the klebecines of 15 standard strains was tested by the method of Edmondson and Cooke (1979).

Plasmid characterisation. (a) *Resistance transfer* from the klebsiella strains to *Escherichia coli* K12 strain J62-2 was tested as described by Datta (1978). (b) *Plasmid visualisation* was by agarose-gel electrophoresis. Single colonies of hospital klebsiellas, or of *E. coli* K12 carrying plasmids derived from them, were lysed on vertical slabs of 0.7% agarose, and plasmid DNA was separated by electrophoresis. Colonies of *E. coli* K12 carrying standard plasmids of known molecular weight were run in parallel. The gels were stained in electrophoresis buffer containing ethidium bromide 5 µl/ml and photographed in ultraviolet light through a Kodak red filter No. 25. The molecular weight of the plasmids from the hospital klebsiellas was calculated from the migration distance relative to that of the standards. The methods were as described by Eckhardt (1978). (c) *Incompatibility groups.* Some of the plasmids identified in this study were classified into incompatibility (Inc) groups by testing their ability or inability to co-exist with standard plasmids of different groups (Datta, 1975).

Transfer of gentamicin resistance to *Klebsiella* strain 10b. The gentamicin R plasmid from the index strain was introduced into the gentamicin-sensitive klebsiella strain 10b from *E. coli* K12 in an attempt to reconstruct the original epidemic strain. The R^+ *E. coli* was grown in mixed broth culture with the klebsiella for 3 h at 37°C and 0.1 ml of the mixture was plated on Simmons citrate agar incorporating gentamicin 3 µg/ml. Colonies developing on this medium were purified by replating on citrate plates. Gentamicin-resistant klebsiella strains so obtained were tested by the typing methods described above.

Mathematical analysis. The methods of numerical analysis used to group the strains, with computer programmes developed at the Microbiology Department, University of Leicester, have been described fully by Sneath and Sokal (1973). Eighty-five characters were recognised from the data and these were scored 1 (+ and resistant) and 0 (– and sensitive). The simple matching (S_{sm}) and Jaccard (S_j) association coefficients were computed between all the strains. These were clustered by the method of unweighted pair-group average linkage.

THE OUTBREAK

This occurred over a period of 3 months during April–July. Thirteen male patients were infected with *Klebsiella* spp. which appeared to be closely similar by biochemical and antibiotic-sensitivity testing. The bacterium was also isolated from two urinals in the affected ward during a period when the urinal pasteuriser was malfunctioning. Details of the association between the strains isolated from patients and urinals are shown in fig. 1. The strains are numbered according to the patient from whom they were isolated. The details are summarised as follows. Each patient was associated epidemiologically with a known positive case. The index patient and 11 others had indwelling urinary catheters. The remaining patient, who developed septicaemia, had been using a contaminated urinal.

The klebsiellas isolated from the two urinals, strains A and B, and from patients 1–10, strains 1–10a, showed identical biochemical characteristics (API profile 5215773) and antibiotic resistance, including resistance to gentamicin, an extremely uncommon feature in the hospital at that time. A later specimen of catheter urine from patient no. 10 yielded a klebsiella, strain 10b which, although similar biochemically and with multiple antibiotic resistance, was

FIG. 1.—Chronological order of isolation of *klebsiella* "outbreak" strains. — = Patient in hospital ward; — — = patient at home; ▼ = isolation of gentamicin-resistant strain; ◻ = isolation of gentamicin-sensitive strain; CSU = catheter specimen of urine.

sensitive to gentamicin. As the outbreak continued, klebsiellas were isolated from patients 11 and 12 which appeared to be identical with strain 10b. From patient 13, a strain was isolated which although similar in resistance pattern to strains 10b, 11 and 12, showed biochemical differences (API profile 5255773). During the period of the outbreak, klebsiellas were isolated from other parts of the hospital that appeared to be epidemiologically unrelated to the above-mentioned strains; one strain was, however, gentamicin resistant. Some of these strains had been routinely maintained on slopes and were later available as a control group (strains 14–20).

RESULTS

The table lists the results obtained from each typing scheme for all the clinical isolates and for one of the laboratory-constructed gentamicin-resistant isolates, strain 10bGm^R. Results for strain 10a are incomplete because it was accidentally lost.

Biochemistry

The API 20E profile identified all isolates as *K. pneumoniae* except for two which it identified as *K. oxytoca*. Of the 16 epidemiologically related isolates, the first 12, strains A, B and 1–10a, were identical and were *K. edwardsi*, MR negative. Strains 10b and 11 were similar except that they were MR positive. Strain 12 differed from the index strain only in being aerogenic (therefore classified as *K. aerogenes*), and strain 13 differed in being aerogenic, indole positive and fermenting dulcitol (*K. oxytoca*). Of the unrelated isolates, strains 14–19 differed from the index strain in being aerogenic and, in some cases, fermenting dulcitol; strain 20 was also indole positive. Biotypes of all strains were reproducible, with no variation occurring in fermentations or gas production when tests were repeated several times in three laboratories. Bascomb *et al.* (1971) consider precise speciation unjustified. They, for example, suggest that *K. aerogenes/oxytoca/edwardsi* can all be considered to belong to one group, *Klebsiella* taxon I.

Antibiotic resistance

Disc diffusion. Results are listed for resistance to all antibiotics within each of three groups (table I). Strains A, B and 1–13 were resistant to those of the first group, which constituted the initial antibiotic tests. Strains A, B, 1–9 and 12 were resistant to that of the second group. Strains A, B and 1–9 were additionally resistant to gentamicin, sisomicin, streptomycin and spectinomycin, whilst strains 10b–13 were additionally resistant to streptomycin alone. Strain 14 was resistant to the antibiotics tested except nalidixic acid, neomycin, paromomycin and amikacin. Strains 15–20 were sensitive to all antibiotics except ampicillin.

Gentamicin MIC. The strains fell into three resistance categories. The

TABLE
Characterisation of *klebsiellas*

Test	Characteristics of epidemiologically related strains						Characteristics of epidemiologically unrelated (control) strains		
	A, B, 1-9	10a	10b-11	10bGm ^R	12	13	14	15-19	20
BIOCHEMISTRY (characteristic of)									
<i>K. edwardsi</i>	+	+	+	+					
<i>K. aerogenes</i>					+		+	+	
<i>K. oxytoca</i>						+			+
ANTIBIOTIC RESISTANCE									
<i>Disc diffusion</i>									
Sul/Tri/Amp/Nal/Nit	+	+	+	+	+	+	†		
Chloramphenicol	+	NT		+	+		+		
Cep/Mec	+	NT				+	+		
<i>Gentamicin resistance (mg/L)</i>									
> 25							+		
8.5	+	+		+					
0.7			+		+	+		+	+
<i>Inferred aminoglycoside modifying enzymes</i>									
AAC (3')-1	+	NT		+					
ANT (2')							+		
SAdT	+	NT		+			+		
SPT	+	NT	+	+	+	+			
None								+	+
CAPSULE TYPE									
21	+	NT	+	+	+		+		
55						+			+
Others‡								+	
PHAGE TYPE									
1/2/16/17	+	NT							
1/2/13/16, 17			+	+					
1/2					+				
1/2/3/16						+			
2							+		
Others§								+	
2/16									+
KLEBECINE TYPE									
9, 11, 15	+	NT	+	+	+				
1, 2, 3, 4, 5, 8						+	+		
1, 2, 3, 4, 5, 8, 9, 11								+	
Others									+
6									
R-PLASMIDS (mol. wt)									
120	+	NT	+	+					
95/90	+	NT		+	+				
46						+			
Others							+		
Not present								+	+

Sul = sulphonamide, Tri = trimethoprim, Amp = ampicillin, Nal = nalidixic acid, Nit = nitrofurantoin, Cep = cephalothin, Mec = mecillinam.

AAC = aminoglycoside-acetylating enzyme, ANT = aminoglycoside-adenylylating enzyme, SAdT = streptomycin-adenylylating enzyme, SPT = streptomycin-phosphorylating enzyme.

+ = Characteristic present, blank space = tested but characteristic absent, NT = not tested.

* MR positive.

† Sensitive only to nalidixic acid.

‡ 3/8/30/44/54.

§ 2, 7, 16, 17/13/15/0/0.

|| 6, 13/0/15/1, 3/0.

epidemiologically related strains, that were gentamicin resistant, had an MIC of 8.5 mg/L whilst those that were sensitive had an MIC of 0.7 mg/L, as did the control strains. Only strain 14, among the epidemiologically unrelated, was highly resistant (> 25 mg/L).

Aminoglycoside-modifying enzymes. The synthesis of four types of plasmid-determined modifying enzymes was inferred, each giving resistance to some aminoglycosides. The evidence suggests that the strains A, B and 1-9 produced aminoglycoside-acetylating enzyme (3')-1, and streptomycin-adenylylating (SAdT) and streptomycin-phosphorylating (SPT) enzymes. Strains 10b, 11, 12 and 13 produced only SPT. The only gentamicin-resistant

strain among the epidemiologically unrelated group, strain 14, produced aminoglycoside-adenylylating enzyme (2'') and SAdT, whilst the other unrelated strains showed no evidence of producing aminoglycoside-modifying enzymes.

Capsule type

Strains A, B and 1–12 were type 21 as was strain 14. Strains 13 and 20 were type 55. The other strains belonged to a variety of different types.

Bacteriophage type

Strains A, B and 1–9 were lysed by four phages whilst strains 10b and 11 were lysed by one additional phage. Strains 12 and 13 differed from strain 1 in susceptibility to two phages. All these strains were susceptible to lysis by phage 1, whereas none of the other strains 14–20 was susceptible. Strains 15–20 differed by two or more phage susceptibilities.

Klebecine type

Strains A, B and 1–12 were inhibited by klebecines 9, 11 and 15. Strain 13 was inhibited by six different klebecines and strain 14 by these and two others. Strain 17 showed partial overlap with strains A, B and 1–12, whilst the remaining control strains were either inhibited by different klebecines or none at all.

Plasmid characterisation

Lysates of colonies of klebsiella strains A, B and 1–9 each showed two plasmid bands in agarose-gel electrophoresis photographs. Each of these two plasmids was transmissible to *E. coli* K12. The larger, of molecular weight about 120×10^6 , conferred resistance to ampicillin/carbenicillin, tetracycline, chloramphenicol and sulphonamides and also determined lactose fermentation. The smaller, of mol. wt about 90×10^6 , conferred resistance to gentamicin, ampicillin and sulphonamides. In strains 10b and 11 the mol wt- 90×10^6 plasmid was absent but the mol. wt- 120×10^6 R-lac plasmid was present and transmissible; it belonged to group IncC.

From strain 12 a band was seen in the gel representing a plasmid of mol. wt- 95×10^6 . It was not transmissible to *E. coli* K12 on selection with any of the antibacterial drugs to which the klebsiella was resistant. Its relationship to the R-lac plasmid of strains 1–11 could not be tested directly. Its molecular weight was different and it appeared unrelated on the following evidence: R40a is a standard plasmid of group IncC (Datta, 1975) incompatible with the R-lac plasmid. R40a was introduced into strain 12 where it replicated stably and did not lead to elimination of any resistance markers. The plasmid content of strain 13 was different (mol. wt 46×10^6) and determined tetracycline resistance only.

Only one of the control strains was found to carry R plasmids. It was the gentamicin-resistant strain 14, which transferred multiple resistance to *E. coli* K12 including resistance to gentamicin, tobramycin, kanamycin and trimethoprim. Four plasmids were detectable in lysates of this strain, with molecular weights different from those of the epidemic klebsiella strain.

Gentamicin R transfer to strain 10b

When the gentamicin R plasmid of strain 1 was introduced into strain 10b the resulting strain, 10bGm^R, resembled strains A, B and 1–9 in its plasmid content and in most of its resistance pattern but it retained distinguishing characters of its 10b parent, *viz.*, MR positive, sensitivity to cephalothin and mecillinam and susceptibility to typing phage 13. Thus, we failed to reconstitute the identity of the epidemic klebsiella, showing that the difference between strain 10b and strains A, B and 1–9 was not merely the loss of one plasmid.

Mathematical analysis

The groups (clusters) obtained from the Jaccard association coefficient are presented as a dendrogram (figure 2). The results obtained from the simple matching coefficient were similar but because many of the data were from test sets that allowed only one positive result, e.g., capsule and klebecine typing, a coefficient that did not take into account negative matches was more suitable.

DISCUSSION

Identification of sources of infection and their subsequent control or elimination depends on ability to distinguish epidemic from other strains of bacteria. Outbreaks of infection may otherwise be attributed to sources with which they are not connected. Distinction is facilitated by the presence of obvious markers such as multiple antibiotic resistance and the application of a typing scheme that is reproducible and discriminatory, such as *Staphylococcus aureus* bacteriophage typing. No system of similar precision to that of the latter has been developed for gram-negative bacteria other than *Salmonella*. Multiple typing schemes have been used to improve precision (Rennie *et al.*, 1978; Rowe *et al.*, 1980).

In the outbreak described here we identified a group of patients infected by a particular strain of *Klebsiella* that had the uncommon marker of gentamicin resistance. The epidemiological situation, however, became confused when a gentamicin-sensitive strain of *Klebsiella* (10b) was isolated from the second catheter specimen of urine from patient no. 10. This event was soon followed by the isolation of gentamicin-sensitive strains (11, 12 and 13) from three other patients who could epidemiologically have been part of the original outbreak. Two explanations were possible: either these four strains were unrelated klebsiellas that had "strayed" into the epidemic or the sensitive strains were derived from the gentamicin-resistant index strain, presumably by a selection

FIG. 2.—Dendrogram showing percentage similarity of the various klebsiellas examined. = 75% association between epidemic strains and strains 10b, 11 and 12; — — = 56% association between the preceding strains and strains 13 and 14; — • — • — = 50% association between all klebsiellas examined (base line).

process favouring loss of gentamicin resistance. These alternatives seemed to be interesting and of epidemiological importance. We attempted to solve the problem by two distinct lines of investigation. Firstly, we compared the four questionable strains with the epidemic strains and with a set of control strains with as many biological markers as we had available. If the four strains in question were “strays” they would show no more relationship with the epidemic strains than would the control strains. The second approach was to re-create the epidemic strain by introducing the gentamicin-resistance plasmid of the index strain into a sensitive strain (10b). Neither of these approaches provided an unequivocal answer to the problem of origin of these klebsiellas. The battery of typing tests showed the control strains, with one exception, to differ in every feature from the epidemic strains. On the other hand, strains 10b, 11 and 12 showed only a few dissimilarities from the epidemic strain.

However, strains 13 and 14 presented problems. The latter had been isolated from a patient who, to the best of our knowledge, had no association with the other patients in the outbreak; yet it had more similarities and fewer dissimilarities than strain 13, which came from a patient who was in close proximity to other patients in the outbreak. We were unable to resolve this problem in any quantitative way and at this stage were left with the strong impression that strains 10b, 11 and 12 were probably derived from the epidemic strain, strain 13 was probably not, and strain 14 was an anomaly. The other approach to this problem was based on the hypothesis that the differences between the index and the sensitive strains might be explained by loss of one of the two resistance (R) plasmids present in the former. However, introduction of the "lost" gentamicin R plasmid from the index strain into strain 10b, making it resistant to gentamicin and chloramphenicol (10bGm^R), did not reconstitute all the characters of the index strain. Our results indicated that sensitivity to cephalothin or mecillinam, bacteriophage-13 susceptibility, MR reaction and gas production could not simply be attributed to loss or gain of one plasmid. A direct link with the epidemic strain was, therefore, not established.

The results we had obtained seemed to be susceptible to a more precise and quantitative analysis. For example, in interpreting the bacteriophage and klebecine susceptibilities we had been able to apply a value for strains that showed identical patterns to the index strain, but we could not arrive at a satisfactory way of quantitating differences between the strains. Because the situation seemed to be analogous to the problems of microbiological systematics, the use of computer analysis seemed feasible, substituting the various epidemiological markers for the usual markers used in such analyses. Computer taxonomy has developed a way of providing mathematical values for relationships between strains and an experience of interpreting their meaning, and we considered that this technique could be used to interpret our data. The data were, therefore, converted for use by a computer programme based on a standard numerical taxonomy package. The methods have previously not been used for such an epidemiological study. This approach yielded a quantitative relationship between the various klebsiellas analysed. It showed that strains 10b, 11 and 12 shared a high degree of similarity with the epidemic strains (75% correlation) which suggests that they belong to the same species (Bascomb *et al.*, 1971). There is as yet no experience in numerical epidemiological analysis, as applied here, as to the percentage agreement that indicates membership of an original clone. However, it can be seen from figure 2 that the association between the epidemic strains and strains 13 and 14 can be demonstrated only at a much lower level (56%), which did not differ significantly ($p > 0.05$) from the level of association for all the klebsiellas examined (50%). There are, therefore, grounds for believing that strains 10b, 11 and 12 were part of the original outbreak but that they had undergone some genetic change. Bacterial plasmid and chromosomal genes may be expected to undergo variation in mixed populations in the natural environment. Conjugative plasmids and transducing phages transfer chromosomal as well as extrachromosomal DNA, providing opportunities for genetic recombination.

Some selective advantage of a variant, whether mutant or recombinant, must be postulated if it is to emerge from the majority population of the clone from which it came. Extra antibiotic resistance confers an obvious advantage but loss of a plasmid may also be advantageous by sparing cells the extra burden of synthesising plasmid DNA and gene products. Our variant strains all carried less plasmid DNA than the original strain. Gentamicin resistance could have been of no advantage to them because none of the patients was treated with gentamicin, but carriage of the gentamicin R plasmid cannot have been a severe disadvantage because the epidemic strain was retained unchanged for long periods in the absence of gentamicin therapy, e.g., by patients 2 and 4. Thus we do not know what selective advantage(s) resulted in the emergence of variants 10b, 11 and 12, but their occurrence illustrates how typing systems may need to be interpreted during epidemic spread of a bacterial clone. The possible effect of plasmid change on phage type (Threlfall, Ward and Rowe, 1978) or bacteriocine type (Davies, Farrant and Tomlinson, 1968) is known, but variations such as we observed have not, to our knowledge, been reported before. A change from anaerogenesis to gas production (strain 12) was especially unexpected, and a change in specific name (Cowan and Steel, 1974) resulted from what we now interpret as being a minor variation.

This outbreak has provided an interesting example of the difficulties of differentiating epidemic from non-epidemic strains of commonly detected bacteria in the absence of a highly selective and differentiating typing system. Computer analysis of less selective typing data provides a way of investigating such outbreaks.

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